

(19) 

Canadian  
Intellectual Property  
Office

An Agency of  
Industry Canada

Office de la Propriété  
Intellectuelle  
du Canada

Un organisme  
d'industrie Canada

(11) CA 2 233 725

(43) 30.09.1999

(13) A1

(12)

(21) 2 233 725

(51) Int. Cl.:

A61K 047/48, C08B 031/00,  
A61K 038/42

(22) 31.03.1998

(71)

HEMOSOL INC.,  
115 Skyway Avenue, ETOBICOKE, O1 (CA).

ADAMSON, J. Gordon (CA).

(74)

Ridout & Maybee

(72)

(54) COMPLEXES D'AMIDON HYDROXYETHYLES D'HEMOGLOBINE

(54) HEMOGLOBIN-HYDROXYETHYL STARCH COMPLEXES

(57)

Hemoglobin conjugates useful as a hemoglobin-based oxygen carrier are prepared by reacting hemoglobin with oxidized hydroxyethyl starch, and allowing the resultant conjugate to degrade to a lower molecular weight product, after conjugation. The conjugate is then reductively stabilized to form secondary amino bonds between the hemoglobin and the hydroxyethyl starch, and formulated as an HBOC.



(21) (A1) **2,233,725**  
(22) 1998/03/31  
(43) 1999/09/30

(72) ADAMSON, J. Gordon, CA

(71) HEMOSOL INC., CA

(51) Int Cl.<sup>6</sup> A61K 47/48, A61K 38/42, C08B 31/00

(54) **COMPLEXES D'AMIDON HYDROXYETHYLES  
D'HEMOGLOBINE**

(54) **HEMOGLOBIN-HYDROXYETHYL STARCH COMPLEXES**

(57) Hemoglobin conjugates useful as a hemoglobin-based oxygen carrier are prepared by reacting hemoglobin with oxidized hydroxyethyl starch, and allowing the resultant conjugate to degrade to a lower molecular weight product, after conjugation. The conjugate is then reductively stabilized to form secondary amino bonds between the hemoglobin and the hydroxyethyl starch, and formulated as an HBOC.



ABSTRACT OF THE DISCLOSURE

Hemoglobin conjugates useful as a hemoglobin-based oxygen carrier are prepared by reacting hemoglobin with oxidized  
5 hydroxyethyl starch, and allowing the resultant conjugate to degrade to a lower molecular weight product, after conjugation. The conjugate is then reductively stabilized to form secondary amino bonds between the hemoglobin and the hydroxyethyl starch, and formulated as an HBOC.

FIELD OF THE INVENTION

This invention relates to biocompatible oxygen carriers for administration to patients as a supplement for or a partial replacement for whole blood. More specifically, the invention relates to hemoglobin-based oxygen carriers (HBOCs) for administration to mammals as a blood substitute or supplement, and processes for their preparation.

BACKGROUND OF THE INVENTION

Hemoglobin, as the natural oxygen transporter component of blood, is an obvious candidate to form the basis of a blood substitute, e.g. as an aqueous solution. Extensive scientific work has been done and reported, on attempts to provide a satisfactory hemoglobin solution to act as a blood substitute. The chemical properties of hemoglobin outside the red blood cells are, however, markedly different from its properties inside the red blood cells, e.g. as regards its oxygen affinity. The need for some form of chemical modification of hemoglobin to render it suitable for use as a blood substitute has long been recognized and has been quite extensively investigated.

It is well known that hemoglobin comprises a tetramer of four sub-units, namely two  $\alpha$  sub-units each having a globin peptide chain and two  $\beta$  sub-units each having a globin peptide chain. The tetramer has a molecular weight of approximately 64 kilodaltons, and each sub-unit has approximately the same molecular weight. The tetrameric hemoglobin in dilute aqueous solution readily dissociates into  $\alpha$ - $\beta$  dimers, and even further under some conditions to  $\alpha$ -sub-unit monomers and  $\beta$ -sub-unit monomers. The dimers and monomers have too low a molecular weight for retention in the circulatory system of the body, and are filtered by the kidneys for excretion with the urine.

This results in an unacceptably short half life of such a product in the body. Moreover, uncross-linked hemoglobin induces significant nephrotoxicity, so that there is a need to minimize the concentration of uncross-linked hemoglobin in the products. The need for chemical bonding between the sub-units to ensure the maintenance of the tetrameric form ("intramolecular cross-linking") has previously been recognized. Also, the linking together of two or more tetrameric units to form hemoglobin oligomers and polymers of molecular weight greater than 64 kilodaltons ("inter-molecular cross-linking") has also been recognized as desirable in many instances.

Accordingly, one approach to developing HBOCs for clinical use has been intramolecularly cross-linking the hemoglobin units into stabilized tetramers, of molecular weight c. 64 kilodaltons, and oligomerizing these tetramers into oligomers of 2-6 such tetramers, by intermolecular cross-linking. A variety of cross-linking reagents have been proposed for this purpose, including oxidatively ring-opened saccharides such as o-raffinose (U.S. Patent 4,857,636 Hsia and U.S. Patent 5,532,352 Pliura et al., for example), bifunctional imidates such as diethyl-malonimide hydrochloride (U.S. Patent 3,925,344 Muzur), halogenated triazines, divinylsulphones, diisocyanates, glutaraldehyde and other dialdehydes (U.S. Patent 4,001,200 Bonsen et al.), bis-diaspirin esters (U.S. Patent 5,529,719 Tye), bis- and tris-acyl phosphates (U.S. Patent 5,250,665 Kluger et al.) and others.

Another approach to the preparation of HBOCs with appropriate molecular weight for clinical use has been the coupling of hemoglobin to a biocompatible polysaccharide. Such conjugates would have the advantage as compared with cross-linked and oligomerized hemoglobins of requiring lower

quantities of hemoglobin per unit of HBOC, and hence would be more economical to prepare. Thus U.S. Patent 4,064,118 Wong proposes the preparation of a blood substitute or blood extender by chemically coupling hemoglobin with a  
5 polysaccharide material selected from dextran and hydroxyethyl starch of molecular weight from about 5 kDa - 2,000 kDa. Only the use of dextran is exemplified in this patent, however.

Baldwin et al. "Tetrahedron" 37, pp 1723-1726 (1981)  
10 "Synthesis of Polymer-Bound Hemoglobin Samples" describe the chemical modification of dextran and hydroxyethyl starch to form aldehyde-substituted polymers, and their subsequent reaction with hemoglobin, to form soluble, polymer-bound hemoglobin. Whilst the products so formed were capable of  
15 binding oxygen, they are reported as unsuitable for use as blood substitutes, since their oxygen-binding curves were considerably left-shifted, indicating that they have too high an oxygen affinity ( $P_{50}$  too low).

## 20 SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel HBOC.

25 It is a further object of the invention to provide a novel polysaccharide-hemoglobin conjugate useful as an HBOC.

It is a further object to provide a process for preparing a novel polysaccharide-hemoglobin conjugate useful as an HBOC.

30 The process of the present invention uses hydroxyethyl starch as the preferred polysaccharide, and prepares an oxidized version of it, in which at least a portion of the glucose monomeric units are oxidized to present aldehyde  
35 groups (hereinafter "oxidized HES"). The oxidized HES so

formed is then reacted with extracellular hemoglobin, so that the hemoglobin, through primary amine groups of the globin chains reacting with the aldehyde groups of the oxidized starch, covalently binds to the starch through Schiff base linkages. Initially and very rapidly there is formed a product which includes species of very high molecular weight, of the order of 500 kDa or higher, in substantial amounts and a wide molecular weight distribution (128->500 kDa). When this product is allowed to stand under appropriate conditions, in aqueous solution, it transforms over a relatively short period of time (e.g. 4-48 hours depending upon the conditions) to a much lower molecular weight product (90-200 kDa) with a much narrower molecular weight distribution. This product, after chemical reduction to reduce the Schiff base linkages between the hemoglobin and the hydroxyethyl starch to secondary amine bonds, turns out to have properties such as oxygen affinity in the range  $P_{50} = 4$  to 50 mmHg at 37°C, depending on the ligand state of the hemoglobin at the time of conjugation, which makes it eminently suitable as a candidate for a hemoglobin based oxygen carrier for clinical use in mammals. Moreover, the resulting product contains no detectable unreacted hemoglobin which, if present, would dissociate to give  $\alpha\beta$ -dimers suspected of causing renal injury, and no detectable amounts of excessively high molecular weight products (over about 500-600 kDa).

Thus according to the first aspect of the present invention, there is provided a hydroxyethyl starch-hemoglobin conjugate useful as a hemoglobin based oxygen carrier and having an oxygen affinity, expressed as partial pressure of oxygen environment required to maintain 50% oxygen saturation,  $P_{50}$  of = 4-50 mmHg, at 37°C, and containing no detectable residual unbound hemoglobin and no detectable residual amounts of components of molecular weight higher than about 500 kDa, said conjugate having been prepared by reacting hemoglobin

with oxidized HES to form a high molecular weight conjugate complex, and allowing the high molecular weight conjugate complex to degrade by storage in solution at a suitable pH value, readily determinable by simple, routine experiments, and at a temperature from 2°C to about 45°C to form said hydroxyethyl starch-hemoglobin conjugate.

According to another aspect, the present invention provides a process of preparing a hemoglobin based oxygen carrier which comprises reacting oxidized HES with hemoglobin to form a conjugate thereof, allowing the conjugate to stand under conditions which effect molecular weight reduction of the conjugate, stabilizing the conjugate by reduction of Schiff linkages between the starch and the hemoglobin to stable, secondary amine linkages, and recovering a solution of the hydroxyethyl starch-hemoglobin conjugate so formed which has no detectable unbound hemoglobin residue and no detectable product residue of molecular weight greater than about 500-600 kDa.

#### BRIEF REFERENCE TO THE DRAWINGS

Figure 1 is a set of chromatograms of products of Example 1 below;

Figure 2 is a further set of chromatograms, of other products of Example 1 below;

Figure 3 is a set of chromatograms of products of Example 5 below; and

Figure 4 is a set of chromatograms of products of Example 6 below.



DESCRIPTION OF THE PREFERRED EMBODIMENTS

The hemoglobin for use in the process of the present invention is preferably human hemoglobin, derived from red blood cells. However, the invention is applicable also to other types of hemoglobin to form the basis of a blood substitute, such as animal hemoglobins especially bovine hemoglobin, and porcine hemoglobin and the like. Human hemoglobin is currently the preferred choice, to form the basis of a blood substitute for administration to human patients.

The hemoglobin can be recovered and prepared for use in the present invention according to standard, known techniques. Thus, red blood cells are lysed, and cellular debris and stroma are removed therefrom by standard techniques of centrifugation, filtration and the like. Preferably, a solution of hemoglobin with a concentration of 2-14% by weight of hemoglobin is used, to yield a product having the most desirable composition and combination of properties. The purity of the hemoglobin should be as high as practically achievable, to avoid toxicity in the final product. Final purification suitably takes place chromatographically. The displacement chromatography process described in U.S. Patent 5,439,591 Pliura et al. is beneficially used.

Hemoglobin can naturally exist in the tight (T) conformation as normally assumed by deoxyhemoglobin, or in the relaxed (R) conformation as normally assumed by oxyhemoglobin or carbon monoxyhemoglobin. The oxygen binding characteristics of deoxy-hemoglobin are the more desirable characteristics, since this is the conformation naturally assumed by the hemoglobin inside the natural red cells of the blood. It is accordingly preferred to use deoxyhemoglobin in the process of the invention. After conjugation to the

hydroxyethyl starch (HES) with or without prior cross-linking, the deoxy-hemoglobin retains its T-configuration. If, however, one chooses for any reason to start with R-configuration hemoglobin, the preferred process according to the invention stabilizes the hemoglobin into the R-configuration throughout.

Deoxygenation of hemoglobin to form deoxyhemoglobin is preferably conducted by subjecting the hemoglobin solution to treatment with a non-oxygenating gas such as nitrogen, according to known techniques. It is preferred to continue the treatment with a stream of nitrogen, followed by appropriate degassing, for sufficiently long periods of time to effect complete conversion to deoxyhemoglobin in this manner.

The hemoglobin can be reacted with the oxidized hydroxyethyl starch (HES) in its native, non-cross-linked form, or in its cross-linked, 64 kDa tetrameric stabilized form, or in its cross-linked and oligomerized form comprising 64 - >500 kDa adducts. When used in its cross-linked form, the preferred cross-linking reagent for preparing cross-linked and cross-linked-oligomerized hemoglobin is a polyaldehyde derived from the oxidative ring-opening of an oligosaccharide such as raffinose (i.e. o-raffinose). A suitable process for preparation of o-raffinose and for its reaction with hemoglobin is described in the above-mentioned U.S. Patent 5,532,352 Pliura et al., the disclosure of which is incorporated herein by reference. Whilst o-raffinose is the preferred cross-linking reagent for use in this embodiment of the invention, it is by no means limited thereto. Any of the other known Hb cross-linking reagents, such as those mentioned previously, for example trimesoylmethyl phosphate (TMMP) described in U.S. Patent 5,250,665 Kluger et al. can be satisfactorily used. The hydroxyethyl starch (HES) starting

material for use in the present invention suitably has a molecular weight of from about 70 to about 1000 kDa. It is commercially available, in various types and varieties. The type and variety for use in the present invention is not critical. Substantially any of the currently commercially available varieties of HES can be used as the starting material, provided that they have a molecular weight approximately as set out above. Those with a substitution ratio (i.e. number of hydroxyethyl groups to glucose units) of from about 0.5 to 0.7 are particularly suitable.

To prepare the HES for use in the present invention, it is oxidized, so as to create thereon substantial numbers of aldehyde groups. This can be accomplished by a variety of oxidation processes, the preferred one being reaction with a periodate (sodium or potassium). This reaction can take place in aqueous solution at low temperature, e.g. 0-5°C, using an appropriate quantity of sodium periodate, chosen according to the desired degree of oxidation. The reaction is complete in about 1-4 hours. The oxidized HES is suitably recovered and redissolved in water, for conjugation to the hemoglobin.

The conjugation reaction suitably takes place in aqueous solution. The hemoglobin may optionally be cross-linked Hb and/or oligomerized Hb. It may be liganded e.g. with carbon monoxide, (CO-Hb). Lower  $P_{50}$  values in the final products are obtained with CO-Hb, higher values with deoxy Hb. Molar ratios of Hb:oxidized HES can range anywhere from about 0.25:1 to 5:1, but are preferably in the 0.5:1-3:1 approximate range. The reaction best takes place at alkaline pH, e.g. in the range 7.5-9.0, and at room temperatures.

The reaction product formed initially, e.g. after about 1 hour is found on analysis to have a very high molecular weight, with components of molecular weight well in excess of

500 kDa, no matter what the molecular weight of the starting HES may have been. This initial product also contains a broad range of molecular weight products. On storage in aqueous solution, preferably in the approximate pH range 7.2-10, and at or close to room temperature (15°C-30°C), the characteristics of the product change, so that within 48 hours of such storage the product contains no products of molecular weight greater than about 500 kDa, and containing a narrow range of molecular weight products, e.g. 100-200 kDa predominate molecular weight distribution. There is very little, if any, residual 32 kDa species. The amount of 32 kDa species is so small that no special steps for its removal are necessary.

The conjugate so formed requires to be stabilized by reducing the (reversible) Schiff base linkages between the Hb and the HES to stable secondary amine linkages. Borane dimethylamine is the preferred choice as the reducing agent. Other water soluble borane lower alkyl amine reducing agents including but not limited to borane-tert-butylamine, borane-ammonia; borane-dimethylamine; borane-trimethylamine; and borane triethylamine, can also be used. Other useful but less preferred reducing agents are sodium cyanoborohydride and sodium borohydride.

Reduction of the Schiff bases formed during the conjugation, and reduction of any residual unreacted aldehyde groups, most suitably takes place in aqueous solution at a temperature range of 2-25°C, for a period of time from 20-36 hours, preferably 24 hours. The reaction mixture is suitably buffered to pH 7-10, preferably to 8.0-9.5. The molar ratio of reducing agent to the sum of imine and aldehyde groups is in the range 2:1 to 5:1, preferably 2.5:1 to 3.5:1 based on the stoichiometry of reducing agent to aldehyde groups added to initiate cross-linking.

It is preferred to use a final step of diafiltration, to remove residual low molecular weight products such as starch degradation residues, dimethylamino borane residues, salts, buffer residues, etc. Then the product can be mixed with a suitable excipient, to form an HBOC.

The conjugate so prepared exhibits eminently suitable properties for use as the basis of an HBOC. It exhibits low oxygen affinity ( $P_{50} = 20-50$  mmHg) along with a narrow molecular weight distribution of product (MWD 100-200 kDa), with no detectable product of m. wt. 32 kDa under conditions which promote dissociation to  $\alpha\beta$ -dimers, or m. wt. above about 500 kDa. It is believed that on storage under slightly alkaline conditions, pH 7.1-10 for example, in aqueous solution, some degradation of the hydroxyethyl starch occurs, to form this lower molecular weight, relatively homogeneous product.

For storage prior to use, it is suitable to treat the product in solution with carbon monoxide, to protect the hemoglobin in its CO-Hb, liganded form. The solution is then stored under sterile conditions. The carbon monoxide ligand is readily removable by known techniques, immediately prior to use. Alternatively, the product can be stored frozen in its  $O_2$ -liganded form.

Dextran offers an alternative but less preferred polysaccharide to HES for use in the present invention. This can similarly be oxidized to an aldehydic form, conjugated to a hemoglobin species, stored to allow degradation, stabilized by reduction and the resulting conjugate formulated as an HBOC.

The invention is further described for illustrative purposes only, in the following specific, non-limiting

examples.

EXAMPLE 1 - PREPARATION OF OXIDIZED HYDROXYETHYL STARCH

5           9.0 g hydroxyethyl starch (HES) with weight average  
molecular weight (MW) of 450 kDa, having a degree of  
hydroxyethyl substitution of 0.7, was dissolved in 90 mL  
water. 0.49, 0.98 and 1.96 g sodium meta-periodate,  
representing approximately 0.3, 0.6 and 1.2 eq, respectively,  
10 of periodate per mol of vicinal diol present in the HES, were  
added to separate 30 mL aliquots of this solution. After 4  
hours reaction in the dark at 4°C, the solutions were dialyzed  
extensively against chilled water using a 15 kDa molecular  
weight cutoff membrane. Final retentates were lyophilized to  
15 white powders and stored at room temperature. Alternatively,  
the dialyzed oxidized HES solution could be used directly for  
conjugation of Hb. HES with MW of 200 kDa and substitution of  
0.5 was oxidized and prepared in a similar manner. Oxidized  
HES was also prepared by direct oxidation of HES formulated in  
20 0.9% NaCl. Measurements of periodate consumption and final  
aldehyde content indicated that the range of periodate used  
resulted in partial to complete oxidation of all available  
diol groups, and that the degree of oxidation was readily  
controlled by varying the amount of periodate used.

25

EXAMPLE 2 - PREPARATION OF CONJUGATES WITH VARIOUS OXIDIZED  
HES and CO-HEMOGLOBIN

30           The reaction of CO-Hb with various relative ratios of  
oxidized HES was studied. Periodate equivalents for oxidation  
were calculated based on the expected vicinal diol content of  
the HES. In one case, 0.54 g oxidized 450 kDa HES, prepared  
using 1.2 eq periodate as described in example 1, was  
dissolved in 3.0 mL 100 mM HEPES buffer pH 8.1. This HES-CHO  
35 solution was added to carbonmonoxylated hemoglobin (COHb, 200

mg/mL in water) in the following ratios: 0.76 mL HES-CHO:0.041 mL COHb, 0.73:0.078, and 0.61:0.195, giving final Hb concentrations of approximately 10, 20 and 50 mg/mL, respectively. The reactions were allowed to proceed at 22-25°C, at pH 8, and samples were withdrawn at various times for MW determination using a Pharmacia Superdex 200 column (1x30 cm) eluted with 0.5 M MgCl<sub>2</sub> + 25 mM Tris pH 7.2 at 0.4 mL/min. At all three HES:COHb ratios, Hb was completely modified in the first several hours to give species having elution times comparable to polyHb controls with MW greater than 128 kDa and ranging to above the exclusion limit of the column (>500 kDa polyHb). Figure 1 of the accompanying drawings shows the chromatogram derived from the 0.5:1 Hb:HES product, taken at various times and compared with (bottom curve) the poly Hb control. The dashed vertical line represents the elution time of unmodified  $\alpha$ - $\beta$  dimer. During the next 30 hours, the elution times of the product decreased to give species having elution times comparable to polyHb controls with MW of 128 kDa, with no unmodified Hb detectable and no species above the exclusion limit of the column. The pattern of MW evolution and final product MW ranges were similar at all HES:Hb ratios, as with HES oxidized with 0.6 eq periodate. The average MW of conjugates formed during the first several hours was lower when less Hb was used. Similar reactions and results were obtained using oxidized 200 kDa HES as described in Example 1. Average MW of final products were higher when 450 kDa HES was used in comparison to 200 kDa HES. Fig. 2 of the accompanying drawings shows chromatograms of the final products of Hb+HES-CHO for HES 200/0.5 (broken lines) and HES 450/0.7 (solid lines), at different degrees of oxidation as indicated, all at 1:1 Hb:HES-CHO ratios.

### EXAMPLE 3 - EFFECT OF LIGAND STATE ON FINAL P<sub>50</sub>

COHb (55 mg/mL in water) was oxygenated and deoxygenated

by exposure to oxygen then nitrogen, respectively. 200 kDa HES was oxidized using 0.6 eq periodate as described in Example 1, and made up to 60 mg/mL in 100 mM HEPES pH 8.1, and degassed and purged with nitrogen. 2.5 mL of this oxidized HES solution was added to 0.8 mL of the deoxygenated Hb solution, providing 1 eq Hb per mol of initial unoxidized 200 kDa HES. After 48 hours at 22-25°C under nitrogen, the reaction mixture was made 0.3 M in sodium acetate, then 3 eq dimethylamine borane per mole of initial aldehyde were added. After 24 hours, the solutions were charged with CO gas, and exhaustively dialyzed against lactated Ringer's solution. A similar procedure was conducted in which COHb, without removal of the CO ligand, was reacted with 200 kDa HES oxidized by 0.6 eq periodate. Oxygen binding properties were measured for both products using a Hemox-Analyzer at 37°C. Conjugation of deoxygenated Hb resulted in a final  $P_{50}$  of 26 mm Hg. Conjugation of COHb resulted in a final  $P_{50}$  of 4 mm Hg. Both products were non-cooperative.

#### 20 EXAMPLE 4 - USE OF CROSS-LINKED HEMOGLOBIN

200 kDa HES was oxidized by 0.3 eq and 0.6 eq periodate in separate reactions as described in Example 1, and made up to 125 mg/mL in 270 mM sodium bicarbonate pH 8.1. 3.0 mL of each oxidized HES solution was added to separate 1.0 mL aliquots of TMMP-cross-linked Hb (64 kDa cross-linked Hb, U.S. Patent 5,250,665 Kluger et al., 125 mg/mL in water), and likewise to 1.0 mL aliquots of o-raffinose polymerized Hb (64 - >500 kDa Hb polymers, U.S. Patent 5,532,352 Pliura et al., 117 mg/mL), for a total of four reactions, in all cases providing 1 eq Hb per mol of initial unoxidized 200 kDa HES. Both hemoglobin products were in the CO form. After 30 hours reaction at 22-25°C under CO gas, sodium acetate was added to a final concentration of 0.3 M. 3 eq dimethylamine borane per mol of initial aldehyde was then added. After 24 hours, the



reactions were dialyzed (10 kDa MWCO) against water then lactated Ringer's solution at pH 7.4. Oxygen binding properties were then recorded using a Hemox-Analyzer at 37°C.

5 MW distributions of all Hbs were shifted to higher values. With Hb and TM-Hb, it was possible to modify all starting Hb with undetectable void volume material (Superose 12, dissociating conditions) within 48 hr. PolyOR-Hb conjugates contained significant void volume material.  $P_{50}$ s (37°) were :HES + CO-Hb, 4 mmHg; HES + deoxyHb, 16-27 mmHg; 10 HES + Co-TM-Hb, 5-7 mmHg; HES + CO-polyOR-Hb, 5-7 mmHg. All products were non-cooperative.

#### EXAMPLE 5 - VARIATION IN REACTION TIME AND TEMPERATURE

15 The effect of shorter reaction times and lower temperature (12 vs. 22°C) on Hb-HES MWD was studied on a small scale. Oxidized forms of 200 kDa and 450 kDa HES were used.

20 Deoxygenated Hb was used. COHb (50 mg/mL in 75 mM HEPES buffer pH 8.1) was oxygenated and deoxygenated by exposure to oxygen then nitrogen, respectively. Oxidized HES, derived from either 200 or 450 kDa HES using 0.6 or 1.2 eq periodate per mol of vicinal diol, respectively, was dissolved in 100 mM 25 HEPES buffer pH 8.1 to a final concentration of 60 mg/mL, and the solutions were then degassed and purged with nitrogen. 0.253 mL of Hb was combined with 1.6 mL of oxidized 200 kDa HES solution, and 0.498 mL of Hb was combined with 1.4 mL of oxidized 450 kDa HES solution, in both cases providing 1 eq Hb 30 per mol of initial unoxidized 200 kDa or 450 kDa HES. These solutions were allowed to react at 22°C under nitrogen, and identical solutions were prepared and allowed to react at 12°C. MWD were determined at various time points as described in Example 2. Chromatographic profiles are shown in Figure 3.

35

Final MWD was narrower at 22°C for both oxidized HES's, and at longer reaction times for both temperatures. Average MW of the 450 kDa HES product (solid lines) was greater than for the 200 kDa derivative (dashed lines), with the MW difference being larger at the lower temperature. Reactions proceeded more slowly at lower temperature, resulting in greater average MW and wider molecular weight range compared to similar reaction times at higher temperature.

#### EXAMPLE 6 - SCALE-UP

Conjugation of oxidized HES to deoxy Hb was scaled up for in vivo evaluation.

COHb was made up to 125 mg/mL in 100 mM HEPES buffer pH 8.1 and rendered ligand-free by contact with oxygen then nitrogen using a hollow fibre gas exchanger. 47 g of oxidized HES, prepared as in Example 1 using 0.6 eq periodate, was dissolved in 280 mL 100 mM HEPES buffer pH 8.1, then degassed and purged with nitrogen. The oxidized HES solution was then added to the deoxyHb and maintained under nitrogen at 22-25°C with occasional measurement of MWD. Within 16 hours, all Hb was modified and no product eluted at the exclusion limit of the column (Fig. 4). The lowermost curve, presented for comparison purposes, is derived from Hb cross-linked with oxidatively ring-opened raffinose (polyOR-Hb). The reaction was made 0.4 M in sodium acetate, and 36 g dimethylamine borane was added, representing approximately 3 eq borane per mole of initial aldehyde. After 21 hours, the reaction mixture was oxygenated, diafiltered (10 kDa MWCO) against lactated Ringer's solution and adjusted to pH 7.4. The product had a P50 (37°C) = 26 mmHg and was non-cooperative. Low angle laser light scattering analysis of size exclusion chromatographic effluent indicated a MW of 90-210 kDa. No free aldehyde was detectable.

A preliminary analysis of in vivo half-life shows that the Hb-HES product is retained for extensive periods.

## WHAT IS CLAIMED IS:

1. A hydroxyethyl starch-hemoglobin conjugate useful as hemoglobin based oxygen carrier and having an oxygen affinity, expressed as partial pressure of oxygen environment required to maintain 50% oxygen saturation,  $P_{50}$  of 4-50 at 37°C, and containing no detectable residual unbound hemoglobin and no detectable residual amounts of components of molecular weight higher than about 500 kDa, said conjugate having been prepared by reacting hemoglobin with oxidized hydroxyethyl starch to form a high molecular weight conjugate complex, and allowing the high molecular weight conjugate complex to degrade by storage in solution at a suitable pH and at a temperature from 2°C to about 45°C, to form said hydroxyethyl starch-hemoglobin conjugate.

2. A process of preparing a hemoglobin based oxygen carrier which comprises reacting oxidized hydroxyethyl starch with hemoglobin to form a conjugate thereof, allowing the conjugate to stand under conditions which effect molecular weight reduction of the conjugate, stabilizing the conjugate by reduction of the Schiff base linkages, between the starch and the hemoglobin to stable, secondary amine linkages, and recovering a solution of the hydroxyethyl starch-hemoglobin conjugate so formed which has no detectable unbound hemoglobin residue and no detectable product residue of molecular weight greater than about 500-600 kDa.

3. A process as claimed in claim 2 wherein oxidized hydroxyethyl starch is replaced by oxidized dextran.

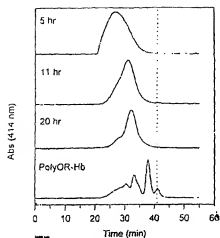


FIG. 1

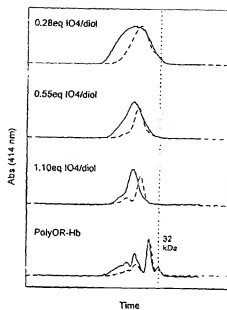


FIG. 2

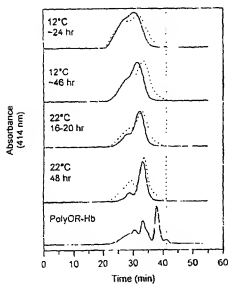


FIG. 3

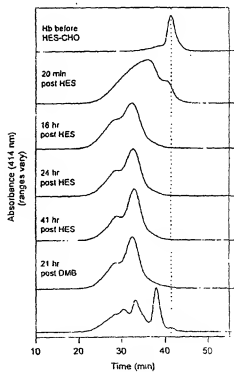


FIG. 4